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(57) Abstract

Isolated polynucleotide molecules encoding mammalian Ship or fragments thereof are provided. The isolated polynucleotide molecules can be used in methods for producing recombinant Ship or biologically active fragments thereof or within methods of diagnosis. Ship polynucleotides of the invention can be used in screens for identifying therapeutic compounds capable of inhibiting or enhancing expression of Ship. Antibodies to Ship can be used to identify mutant Ship molecules in diagnostic methods for Ship-associated diseases such as cancer.

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DNA ENCODING AN SH2-INOSITOL PHOSPHATASE, A SHC-BINDING PROTEIN

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Background of the Invention

Fms is a tyrosine kinase growth factor receptor closely related to the c-Kit, Flt3 and PDGF receptors. While the PDGF receptor is primarily expressed in fibroblasts and endothelial cells, Fms, Kit and Flt3 are expressed in a lineage dependent fashion in hematopoietic cells. Flt3 is expressed in very early progenitor cells, Kit in intermediate developing cells as well as some mature lineages, and Fms is expressed in later stages of hematopoiesis when monocyte/macrophage development occurs. These receptors control signals for growth, survival, differentiation, and expression of mature cell functions.
Thus, Fms and its signal transduction pathways appear to be integral to the overall process of hematopoietic cell development.

The initial stages after Fms activation in the Fms signal transduction pathways have been characterized. After binding of homodimeric macrophage colony-stimulating factor (M-CSF, or CSF-1), the receptor is dimerized, transphosphorylated on specific cytoplasmic tyrosines and is down-modulated by internalization and degradation. Receptor autophosphorylation sites serve as docking sites for binding

of signal transduction molecules which transmit and amplify signals downstream, and additional cytoplasmic proteins are tyrosine phosphorylated, although it is not clear what kinases are involved in this process.

A tyrosine phosphorylated protein of 145-150 kDa has been identified in a number of hematopoietic cell lines after stimulation with a variety of lymphokines. Antigen receptor cross-linking in B-cells, IL-3, Steel factor and erythropoietin stimulation of erythroid cells and megakaryocytes or M-CSF stimulation of monocytes and erythroid cells, induces the tyrosine phosphorylation of a 145 kDa protein that is found in a complex with Shc, Grb2 and Sos. Although in all of these systems the same molecules form complexes, the interaction mechanism between Shc and p145 is not well understood, due either to different p145-150 molecules or to different phosphorylation patterns from stimulation of different cells with different lymphokines.

Although Fms induces the tyrosine phosphorylation of p150 and its association with Shc and Grb2/Sos in hematopoietic cells, this tyrosine phosphorylated protein has not been observed in Fms-expressing fibroblasts after M-CSF stimulation. In contrast, PDGF stimulation of fibroblasts induces the phosphorylation of a 145 kDa protein that associates with the Shc-PTB domain. Whether these two molecules are different proteins or whether an intermediate molecule required in Fms signaling for p150 activation is not present in fibroblasts is unknown.

Activation of the EGF receptor, PDGF receptor and insulin receptor results in the phosphorylation of Shc and Ras activation. By analogy, the Fms-mediated phosphorylation of Shc and its interaction with Fms and Grb2/Sos suggests that M-CSF stimulation of myeloid cells could activate Ras. The Fms proto-oncogene has been implicated in myeloid leukemias and oncogenic versions of the c-Fms protein are encoded by two different feline sarcoma viruses.

What is needed is a better understanding of the Fms signal transduction pathway and its involvement in

oncogenesis, and therapeutic and diagnostic approaches based thereon. Quite surprisingly, the present invention addresses these and other related needs.

Summary of the Invention

The present invention provides isolated and substantially pure preparations of mammalian SHIP and biologically active fragments thereof. The invention also provides antibodies to SHIP, in the form of antisera and/or monoclonal antibodies.

In another aspect the invention provides the ability to produce SHIP and polypeptides or fragments thereof by recombinant means, preferably in cultured eukaryotic cells. The expressed SHIP or fragments thereof may or may not have the biological activity of corresponding native SHIP. Accordingly, isolated and purified polynucleotides are described which code for SHIP and fragments thereof, where the polynucleotides may be in the form of DNA, such as cDNA, or RNA. Based on these sequences probes may be used to hybridize and identify these and related genes which encode SHIP. The probes may also be used to identify mutations in SHIP for diagnostic purposes. The probes may be full length cDNA or as small as from 14 to 25 nucleotides, more often though from about 40 to about 50 or more nucleotides.

In related embodiments the invention concerns DNA constructs which comprise a transcriptional promoter, a DNA sequence which encodes SHIP or a fragment thereof, and a transcriptional terminator, each operably linked for expression of the SHIP.

In other embodiments, the polynucleotide molecules encoding SHIP and antibodies to SHIP may be used to identify mutations in SHIP that are associated with certain diseases, such as cancer. As such the invention relates to methods for diagnosis, where the polynucleotides molecules and antibodies

are used to detect the presence of SHIP mutations in a biological sample. For example, an antibody which specifically binds SHIP or a SHIP mutant is incubated with the sample under conditions conducive to immune complex formation, which complexes are then detected, typically by means of a label such as an enzyme, fluorophore, radionuclide, chemiluminescer, particle, or a second labeled antibody. Thus, means are provided for immunohistochemical staining of tissues, including tumor biopsies.

Description of the Specific Embodiments

SH2-Inositol Phosphatase ("SHIP") is a protein capable of binding to Shc, a transforming protein with an SH2 domain that is implicated in mitogenic signal transduction. Activation of tyrosine kinase receptors, such as Fms, the EGF receptor, insulin receptor and PDGF receptor results in Shc phosphorylation and Ras activation. Ras activation to its GTP-bound form is facilitated by the nucleotide exchange factor Sos. Grb2, a 23 kD protein composed entirely of one SH2 domain flanked by two SH3 domains, binds through both of its SH3 domains to proline-rich sequences of Sos and through its SH2 domain to other tyrosine-phosphorylated proteins such as activated EGF receptor or tyrosine kinase substrates such as Shc. Interactions of Grb2 with any of these tyrosine-phosphorylated proteins results in the translocation of Sos to the plasma membrane, where it stimulates Ras to exchange GDP for GTP. Upon M-CSF stimulation of cells expressing the Fms receptor, Shc proteins are phosphorylated on tyrosine and participate in a complex formation with Fms, Grb2, and SHIP by directly interacting with Grb2 and SHIP.

As part of the present invention it has been determined that the structure and function of SHIP is consistent with that of an inositol phosphate 5-phosphatase (IP5P). Purified SHIP has an apparent molecular weight of 150

kD as determined by SDS PAGE, and a calculated molecular weight of 133,438, as determined from an open reading frame translating for an 1190 amino acid protein. For consistency, the molecular weight of SHIP is referred to herein as 150 kD. The present invention provides representative polynucleotide sequences encoding SHIP. As discussed in more detail herein, sequence analysis of a representative SHIP cDNA demonstrates that SHIP contains an SH2 (src homology-2) domain at its N-terminus, a C-terminal region with two NPXY [SEQ ID NO:14] motifs that are known to bind phosphotyrosine binding (PTB) domains and numerous proline rich clusters with motifs that are known to bind SH3 (src homology-3) domains.

It is an object of the present invention to provide isolated polynucleotide molecules encoding SHIP. It is also an object of the present invention to provide methods for producing SHIP from recombinant host cells. An additional object of the present invention is to provide methods for detecting mutations in SHIP at its genetic locus that are implicated in disease states such as cancer. An additional feature of the present invention are purified antibodies directed towards SHIP that are useful in detecting the presence of SHIP and/or SHIP mutants in biological samples such as tumor biopsies. Such isolated molecules are those that are separated from their natural environment and encompass oligonucleotides, cDNA and genomic clones.

The present invention provides the advantage that SHIP encodes a protein involved in signal transduction and the control of cell proliferation. The nucleotide sequences encoding the protein appear related to sequences encoding the human 51C gene product associated with Fanconi's anemia, the X-linked Lowe's oculocerebrorenal syndrome (OCRL) gene, and inositol polyphosphate 5-phosphatases. The symptoms associated with these genes are considered to be related to errors in maintaining DNA fidelity at replication. SHIP may thus function in cell cycle checkpoint control, in that the activation of SHIP following M-CSF stimulation of Fms may positively regulate a checkpoint pathway for the transit of

cells from G1 to the S phase, although this is offered by way of possible explanation and not limitation. Reduced control over this stage, by way of mutations in SHIP, may result in unregulated cell growth and proliferation.

To understand the roles of Shc and SHIP in signal transduction their interaction was characterized and cDNA encoding SHIP was cloned. In hematopoietic cells, as in fibroblasts, tyrosine phosphorylated SHIP binds to the Shc-PTB domain. A glutathione S transferase (GST)-fusion protein containing the N-terminal portion of murine Shc (aa 48-209) competed for SHIP binding in Shc immunoprecipitation experiments. When the fusion protein was incubated with cell lysates from M-CSF stimulated hematopoietic cells expressing exogenous murine Fms, it readily bound tyrosine phosphorylated SHIP with high affinity. This association was only partially interrupted by the addition of 100 mM phenylphosphate, a phosphotyrosine analog, indicating a high affinity interaction. In addition, such complexes contained very low levels of Shc, consistent with the observation that the predominant interaction of Shc with SHIP was that of the Shc-PTB domain interacting with a phosphorylated tyrosine in an NPXY [SEQ ID NO:14] motif.

In hematopoietic cells, SHIP was found in large complexes containing at least Shc, Grb2/Sos and PI3 kinase. Mutations which abolish Fms' interactions with PI3 kinase (Y721F) or Grb2/Sos (Y697F) did not alter Fms functions, i.e., survival, proliferation or differentiation, and none of the tyrosine to phenylalanine mutations on Fms affected p150 phosphorylation or its association pattern with Shc, Grb2/Sos and PI3K. Such complexes indicate an alternative Fms signaling pathway.

To further characterize SHIP, the phosphorylated protein was purified and amino acid sequence information obtained for Lys C-generated peptides. Degenerate oligonucleotides based on these peptides were designed for use in PCR technology to screen cDNA libraries, but were not successful in generating cDNA fragments corresponding to SHIP.

A modified yeast two-hybrid system was combined with the high affinity binding of the Shc-PTB domain for phosphorylated SHIP to clone a cDNA encoding for SHIP.

The yeast two hybrid system includes a LexA fusion vector pBTM116 which permits the in-frame fusion of LexA to a protein of interest ("bait") and a VP16 fusion vector, pVP16, into which cDNA fragments are ligated to create a library. The LexA:bait can bind to the LexA operator but is unable to activate transcription because it cannot associate with RNA polymerase. VP16 can associate with RNA polymerase but does not recognize the LexA operator. Accordingly, cotransformation of pBTM116:bait and pVP16 does not activate *HIS3* transcription. However, a bait binding partner fused to VP16 provides a bridge between LexA and VP16 and consequently activates *HIS3* expression. Yeast cotransfected with pBTM116:bait and pVP16:bait-binding partner grows when plated on selective medium lacking histidine. Although this system can be utilized to identify protein:protein interactions, it is limited in identifying protein:phosphotyrosine interactions because yeast do not express active tyrosine kinases. Thus, the pBTM116 vector was modified to express a tyrosine kinase, in addition to the LexA:bait fusion protein.

In the modified system employed in the present invention, the PTB domain of Shc was fused to the LexA protein (LexA-PTB) as bait, and the EGF receptor C-terminal tail was fused to the VP16 protein as a target for testing the ability of kinases to phosphorylate the known PTB-binding motif (NPXY) in the EGF receptor cytoplasmic tail (EGFRT) domain and bind the Shc-PTB domain. Among three tyrosine kinases tested the PDGF receptor cytoplasmic domain was the most effective in phosphorylating the target EGF cytoplasmic domain with resultant VP16-EGFRT binding, with growth of β-Gal positive cells. Therefore, the two hybrid screen used the pBTM116/PDGFR vector with the Shc-PTB domain (aa 48-209) as the bait, and a VP16-EML library. Transformants that were positive for transcriptional activation were "cured" to allow

loss of the pBTM116 vector and mated with a panel of new lexA:baits to ensure specificity.

DNA sequence analysis on a subset of the clones that passed all the criteria for growth and β-galactosidase activity showed that all fourteen clones analyzed contained the NPXY motif, and ten appeared to be derived from the same gene. Northern blot analysis of polyA selected RNA from FDC-P1 cells using the cDNA insert (1.1 kb) from one of the clones (EML-11) identified in the yeast two hybrid screen as a probe indicated a single mRNA species of approximately 5.0 kb which could accommodate the translation of a 150 kDa protein.

The same cDNA insert (EML-11) was used as a probe to screen a polyA cDNA library constructed from FDC-P1 clone19 and Mac11 cells. Two of the 10 clones identified contained inserts of approximately 5.0 kb and one of these clones (clone pBK-CMV-150.8) was chosen for DNA sequencing in both orientations. The clone pBK-CMV-150.8 contained a 4863 nucleotide insert with an open reading frame (ORF) translating for 1190 amino acid protein with a calculated molecular weight of 133,438. Translation probably starts with the methionine at position 1 since one of the peptides assigned to SHIP after Lys C digestion contained the sequence PAMVPGWN [SEQ ID NO:17] which corresponds to amino acids 2-9 of SEQ ID NO:13 and contains the methionine at position 4. Furthermore, in the same reading frame all eleven Lys C peptides derived from the native protein were identified, distributed throughout the ORF including a peptide (GRDYRDNTELP; SEQ ID NO:16) only 19 amino acids before the stop codon.

The N-terminus of SHIP is occupied by an SH2 domain and the C-terminal region contains two NPXY motifs (NPNY (amino acids 914-917 of SEQ ID NO:13) and NPLY (amino acids 1017-1020 of SEQ ID NO:13) that bind PTB domains. From the 10 clones identified with the two hybrid screen, three contained only the NPNY motif and seven clones contained both the NPNY and NPLY motifs. This indicates that at least the tyrosine in the NPNY context is phosphorylated and binds to the Shc-PTB domain. The C-terminal one-fourth of the molecule contains

numerous proline rich clusters of which at least three appear to be motifs that are known to bind SH3 domains.

An appreciable proportion of ATP or GTP binding proteins share a conserved motif or P loop within a glycine rich region that is believed to interact with one of the phosphate groups of the nucleotide. SHIP contains such a motif (GQPLHGKS) [SEQ ID NO:15] only seven amino acids after the NPNY [SEQ ID NO:13] sequence.

The SHIP cDNA insert from clone pBK-CMV-150.8 was used to identify and clone a partial human cDNA encoding human SHIP. The missing 5' coding sequence of human SHIP were obtained by PCR amplification of SHIP sequences from a human HL-60 library. The human SHIP contains a 3566 bp coding seuqence and a deduced amino acid sequence of 1189 amino acids [SEQ ID NOS:26 and 27].

The central portion of SHIP exhibits 55% identity and 73% similarity to the human 51C gene product. The 51C cDNA was cloned by its ability to complement the Fanconi anemia group C complementation (FACC) gene for hypersensitivity to DNA-damaging agents. The FACC gene encodes a 60 kDa protein and antibodies to this protein also recognize a related 150 kDa protein. The similarity between SHIP and 51C also extends to the nucleotide level.

SHIP also shares homology with the X-linked Lowe's oculocerebrorenal syndrome gene (OCRL). SHIP, 51C, and the OCRL protein all share amino acid sequence homology with proteins involved in inositol metabolism which remove the 5' phosphate of inositol (1,4,5) and/or inositol (1,3,4,5). SHIP's homology with these proteins is not as extensive as with 51C, numerous smaller domains are conserved among SHIP, 51C, OCRL, and inositol polyphosphate-5-phosphatases (Table I, below).

Thus, M-CSF activation of Fms leads to the downstream tyrosine phosphorylation within at least the NPNY motif of a 150 kDa inositol polyphosphate-5'-phosphatase-like protein, SHIP, and its association at this site with Shc through the amino-terminal PTB domain. The SH2 domain of SHIP

permits additional interactions with tyrosine phosphorylated proteins, and proline-rich sequences in the carboxyl domain can couple to proteins containing SH3 domains. SHIP is tyrosine phosphorylated and associates with Shc after stimulation of responsive erythroid cells with either Epo or M-CSF. SHIP or a related tyrosine phosphorylated protein is detected after M-CSF stimulation of Fms-expressing myeloid cells after activation of the EpoR and IL-3R. Also, SHIP participates in signaling by IL-3 and M-CSF in FD(Fms) cells (and weakly by GM-CSF stimulation). In contrast, the p145 tyrosine phosphorylated and Shc-PTB-binding protein in PDGF-stimulated fibroblasts differs from SHIP because no protein with these properties was tyrosine phosphorylated after M-CSF stimulation of Rat2(Fms) fibroblast cells.

The role of an inositol polyphosphate-5'-phosphatase in a Fms signaling pathway indicates a function in Ca^{2+} signaling. While offered by way of possible explanation and not limitation, as inositol-1,4,5-triphosphate increases the intracellular concentration of Ca^{2+}, SHIP may terminate that signal. Additionally, 5'-phosphorylated phosphoinositides can bind to pleckstrin homology domains and activate such molecules as the Akt/PKB kinase, and again SHIP may regulate this process.

The genes to which SHIP shows homology are linked to several disease states in humans. The 51C gene was isolated by its complementation of the DNA repair defect in cells from a Fanconi anemia patient. This defect also predisposes individuals to an increased incidence of myeloid leukemia. The mutated OCRL protein, linked to the oculocerebrorenal syndrome of Lowe, is characterized by congenital cataracts, mental retardation, and defective renal tubular function. The broad spectrum of symptoms in each condition indicates an error in maintaining DNA fidelity at replication. A number of other hereditary syndromes also fall into this same category: ataxia telangiectasia (AT), Bloom's, Werner's (premature aging), and xeroderma pigmentosum. Part of the AT gene has been identified as encoding a large protein

with a carboxyl terminal domain suggesting PI 3'-kinase activity. A related gene, MEC1, also contains a domain related to PI 3'-kinase, and both genetic and biochemical studies indicate that these proteins function as controls for cell cycle checkpoints. The involvement of inositol metabolism in TOR and $p70^{S6k}$ activation, their regulation of cell cycle progression form G1 to S phase, and the related disease states indicate that SHIP activity functions in cell cycle checkpoint control. The activation of SHIP following M-CSF stimulation of Fms may positively regulate a checkpoint pathway for the transit of cells from G1 to the S phase. Reduced control over this stage is consistent with observations that Fms activation in FDC-P1 cells results in a gradual increase in factor-independent cells. Thus, SHIP fits into a group of proteins and their associated diseases that indicates a relationship to Ca^{2+} metabolism and/or events that prevent the propagation of DNA damage by controlling transition at the G1/S boundary of the cell cycle.

Accordingly, in one embodiment the present invention provides representative polynucleotide molecules and amino acids sequences encoding SHIP. Sequences encoding SHIP include those sequences that result in minor variations in amino acid sequence, such as those due to genetic polymorphisms, allelic variations, differences between species and those in which blocks of amino acids have been added, altered or replaced without substantially altering the biological activity of the proteins.

As noted above, the present invention provides isolated and purified polynucleotide molecules encoding SHIP. Polynucleotide molecules encoding SHIP are capable of hybridizing under stringent conditions to an oligonucleotide of 14 or more contiguous nucleotides of SEQ ID NO:12 or SEQ ID NO:26, and the complementary strand of SEQ ID NO:12 or SEQ ID NO:26.

The polynucleotide molecules encoding Ship include sequences substantially identical to the sequences of SEQ ID NO: 12 or SEQ ID NO:26 The terms "substantially corresponds

to", "substantially homologous", or "substantial identity" as used herein denotes a characteristic of a nucleic acid sequence, wherein a nucleic acid sequence has at least about 70 percent sequence identity as compared to a reference sequence, typically at least about 85 percent sequence identity, and preferably at least about 95 percent sequence identity as compared to a reference sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 25 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, the reference sequence is at least 18 nucleotides long, typically at least about 30 nucleotides long, and preferably at least about 50 to 100 nucleotides long. "Substantially complementary" as used herein refers to a sequence that is complementary to a sequence that substantially corresponds to a reference sequence.

The disclosed sequences may be used to identify and isolate SHIP polynucleotide molecules from suitable mammalian hosts such as canine, ovine, bovine, equine, caprine, or lagomorph, or avian hosts. Nucleotide sequences 5' or 3' of sequence domain encoding the inositol phosphate 5-phosphatase (IP-5P) may be particularly useful in identifying polynucleotide molecules encoding SHIP from other species. Complementary DNA molecules encoding SHIP may be obtained by constructing a cDNA library mRNA from, for example, myeloid cells, monocytes, and macrophages. DNA molecules encoding SHIP may be isolated from such a library using the disclosed sequences in standard hybridization techniques (e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989 and Bothwell, Yancopoulos and Alt, Methods for Cloning and Analysis of Eukaryotic Genes, Jones and Bartlett Publishers, Boston, MA 1990) or by amplification of sequences using polymerase chain reaction (PCR) amplification (e.g, Loh et al. Science 243: 217-222, 1989; Frohman et al., Proc. Natl. Acad. Sci. USA 85:

8998-9002, 1988; and Erlich (ed.), PCR Technology: Principles and Applications for DNA Amplification, Stockton Press, 1989; and U.S. Patent No. 4,683,195, which are incorporated by reference herein in their entirety). In a similar manner, genomic DNA encoding SHIP may be obtained using probes designed from the sequences disclosed herein. Upstream regulatory regions of SHIP may be obtained using the same methods. Suitable PCR primers are between 7-50 nucleotides in length, more preferably between 14 and 25 nucleotides in length. Polynucleotide molecules encoding SHIP may be isolated using standard hybridization using probes of at least about 7 nucleotides in length and up to and including the full coding sequence.

The choice of hybridization conditions will be evident to one skilled in the art and will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of relatedness between the sequences. Methods for hybridization are well established in the literature; See, for example: Sambrook, ibid.; Hames and Higgins, eds, Nucleic Acid Hybridization A Practical Approach, IRL Press, Washington DC, 1985; Berger and Kimmel, eds, Methods in Enzymology, Vol. 52, Guide to Molecular Cloning Techniques, Academic Press Inc., New York, NY, 1987; and Bothwell, Yancopoulos and Alt, eds, Methods for Cloning and Analysis of Eukaryotic Genes, Jones and Bartlett Publishers, Boston, MA 1990; which are incorporated by reference herein in their entirety. One of ordinary skill in the art realizes that the stability of nucleic acid duplexes will decrease with an increased number and location of mismatched bases; thus, the stringency of hybridization may be used to maximize or minimize the stability of such duplexes. Hybridization stringency can be altered by: adjusting the temperature of hybridization; adjusting the percentage of helix-destabilizing agents, such as formamide, in the hybridization mix; and adjusting the temperature and salt concentration of the wash solutions. In general, the stringency of hybridization is adjusted during the post-hybridization washes by varying the

salt concentration and/or the temperature. Stringency of hybridization may be reduced by reducing the percentage of formamide in the hybridization solution or by decreasing the temperature of the wash solution. High stringency conditions may involve high temperature hybridization (e.g., 65-68°C in aqueous solution containing 4-6X SSC, or 42°C in 50% formamide) combined with high temperature (e.g., 5-25°C below the T_m) and a low salt concentration (e.g., 0.1X SSC). Reduced stringency conditions may involve lower hybridization temperatures (e.g., 35-42°C in 20-50% formamide) with intermediate temperature (e.g., 40-60°C) and washes in a higher salt concentration (e.g., 2-6X SSC). Moderate stringency conditions, which may involve hybridization at a temperature between 50°C and 55°C and washes in 0.1x SSC, 0.1% SDS at between 50°C and 55°C, may be used to identify clones encoding polynucleotide molecules encoding SHIP from other species.

Recombinant SHIP may be produced by inserting an isolated DNA molecule encoding SHIP into a suitable expression vector, which is in turn used to transfect or transform a suitable host cell. Suitable expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a polynucleotide molecule of interest in a host cell. Representative expression vectors may include both plasmid and/or viral vector sequences. Suitable vectors include retroviral vectors, vaccinia viral vectors, CMV viral vectors, BLUESCRIPT (Stratagene Cloning Systems), baculovirus vectors, and the like. Promoters capable of directing the transcription of a cloned gene or cDNA may be inducible or constitutive promoters and include viral and cellular promoters. For expression in mammalian host cells, suitable viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41: 521-530, 1985) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1: 854-864, 1981). Suitable cellular promoters for expression of proteins in mammalian host cells include the mouse metallothionien-1 promoter (Palmiter et al.,

U.S. Patent No. 4,579,821), a mouse Vk promoter (Bergman et al., Proc. Natl. Acad. Sci. 81: 7041-7045, 1983; Grant et al. Nucleic Acid. Res. 15: 5496, 1987) and tetracycline-responsive promoter (Gossen and Bujard, Proc. Natl. Acad. Sci.USA 89: 5547-5551, 1992 and Pescini et al., Biochem. Biophys. Res. Comm. 202: 1664-1667, 1994). Also contained in the expression vectors is a transcription termination signal located downstream of the coding sequence of interest. Suitable transcription termination signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982), the polyadenylation signal from the Adenovirus 5 e1B region and the human growth hormone gene terminator (DeNoto et al., Nucleic Acid. Res. 9: 3719-3730, 1981). Mammalian cells for example may be transfected by a number of methods including calcium phosphate precipitation (Wigler et al., Cell 14: 725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7: 603, 1981; Graham and Van der Eb, Virology 52: 456, 1973); lipofection, microinjection and electroporation (Neumann et al., EMBO J. 1: 8410845, 1982). Mammalian cells can be transduced with virus vectors such as SV40, CMV and the like. In the case of viral vectors, cloned DNA molecules may be introduced by infection of susceptible cells with viral particles. Retroviral vectors may be preferred for use in expressing SHIP in mammalian cells particularly if SHIP is used for gene therapy (for review, see, Miller et al., Meth. Enzymol. 217: 581-599, 1994; which is incorporated herein by reference in its entirety). It may be preferable to use a selectable marker to identify cells that contain the cloned DNA. Selectable markers are generally introduced into the cells along with the cloned DNA molecules and include genes that confer resistance to drugs, such as neomycin, hygromycin and methotrexate. Selectable markers may also complement auxotrophies in the host cell. Yet other selectable markers provide detectable signals, such as beta-galactosidase to identify cells containing the cloned DNA molecules. Selectable markers may be amplifiable. Such

amplifiable selectable markers may be used to amplify the number of sequences integrated into the host genome.

As would be evident to one of ordinary skill in the art, the polynucleotide molecules of the present invention may be expressed Saccharomyces cerevisiae, filamentous fungi, and E. coli. Methods for expressing cloned genes in Saccharomyces cerevisiae are generally known in the art (see, "Gene Expression Technology," Meth. Enzymol., vol. 185, Goeddel (ed.), Academic Press, San Diego, CA, 1990 and "Guide to Yeast Genetics and Molecular Biology," Meth. Enzymol., Guthrie and Fink (eds.), Academic Press, San Diego, CA, 1991; which are incorporated herein by reference). Filamentous fungi (e.g., strains of Aspergillus) may also be used to express the proteins of the present invention. Methods for expressing genes and cDNAs in cultured mammalian cells and in E. coli is discussed in detail in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Ed., Cold Spring Harbor, NY, 1989; which is incorporated herein by reference). As would be evident to one skilled in the art, one could express the protein of the instant invention in other host cells such as avian, insect and plant cells using regulatory sequences, vectors and methods well established in the literature.

The SHIP polynucleotides of the present invention may be useful in gene therapy protocols in which it is desirable to overexpress SHIP, such as in protocols designed to re-establish checkpoint control in cancer or other diseases associated with loss of checkpoint control.

SHIP proteins produced according to the present invention may be purified using a number of established methods such as affinity chromatography using anti-SHIP antibodies coupled to a solid support. Additional purification may be achieved using conventional purification means such as liquid chromatography, gradient centrifugation and gel electrophoresis among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, NY, 1982, which is incorporated herein by reference) and may be applied to the

purification of recombinant SHIP described herein. Thus, SHIP is provided isolated from its natural cellular environment, and substantially free of other cellular proteins. Purified SHIP is also provided, where substantially pure SHIP of at least about 50% is preferred, at least about 70-80% is more preferred, and 95-99% or more homogeneity most preferred. Once purified, partially or to homogeneity, as desired, the recombinant SHIP or native SHIP may be used to generate antibodies, diagnostically in assay procedures, etc.

Antisense SHIP nucleotide sequences may be used to block overexpression of SHIP or expression of mutant forms of SHIP. The use of antisense oligonucleotides and their applications have been reviewed in the literature (see, for example, Mol and Van der Krul, eds., Antisense Nucleic Acids and Proteins Fundamentals and Applications, New York, NY, 1992; which is incorporated by reference herein in its entirety). Suitable antisense oligonucleotides are at least 11 nucleotide in length and up to and including the upstream untranslated and associated coding sequences. As will be evident to one skilled in the art, the optimal length of antisense oligonucleotides is dependent on the strength of the interaction between the antisense oligonucleotides and their complementary sequence on the mRNA, the temperature and ionic environment in which translation takes place, the base sequence of the antisense oligonucleotide, and the presence of secondary and tertiary structure in the mRNA and/or in the antisense oligonucleotide. Suitable target sequences for antisense oligonucleotides include intron-exon junctions (to prevent proper splicing), regions in which DNA/RNA hybrids will prevent transport of mRNA from the nucleus to the cytoplasm, initiation factor binding sites, ribosome binding sites, and sites that interfere with ribosome progression. A particularly preferred target region for antisense oligonucleotide is the 5' untranslated region of the gene of interest. Antisense oligonucleotides may be prepared by the insertion of a DNA molecule containing the target DNA sequence into a suitable expression vector such that the DNA molecule

is inserted downstream of a promoter in a reverse orientation as compared to the gene itself. The expression vector may then be transduced, transformed or transfected into a suitable cell resulting in the expression of antisense oligonucleotides. Alternatively, antisense oligonucleotides may be synthesized using standard manual or automated synthesis techniques. Synthesized oligonucleotides may be introduced into suitable cells by a variety of means including electroporation, calcium phosphate precipitation microinjection. The selection of a suitable antisense oligonucleotide administration method will be evident to one skilled in the art. With respect to synthesized oligonucleotides, the stability of antisense oligonucleotides-mRNA hybrids may be increased by the addition of stabilizing agents to the oligonucleotide. Stabilizing agents include intercalating agents that are covalently attached to either or both ends of the oligonucleotide. Oligonucleotides may be made resistant to nucleases by, for example, modifications to the phosphodiester backbone by the introduction of phosphotriesters, phosphonates, phosphorothioates, phosphoroselenoates, phosphoramidates or phosphorodithioates. Oligonucleotides may also be made nuclease resistant by the synthesis of the oligonucleotides with alpha-anomers of the deoxyribonucleotides.

The invention also provides SHIP protein fragments and peptides. The SHIP peptides and fragments of the invention are desirably biologically active. The peptides can be prepared via chemical synthesis, as described hereinbelow, or by recombinant DNA technology, or as fusion proteins, and the like. Desirably, the peptide will be as small as possible while still maintaining substantially all of the reactivity of a larger peptide. The subject peptides have an amino acid sequence encoded by a nucleic acid which hybridizes under stringent conditions with an oligonucleotide of 15 or more contiguous nucleotides of SEQ ID NO:12 or SEQ ID NO:27. It will be understood that the peptides and fragments of the present invention or analogs thereof which have Shc binding or

other reactivity may be modified from a native SHIP sequence as necessary to provide other desired attributes, e.g., improved binding or inhibitory activity (e.g., increased competition with native protein), improved adsorption to a solid phase, etc. For instance, the peptides may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Usually, the sequence of the peptide will not differ by more than about 20% from the native SHIP sequence, except where additional amino acids may be added at either terminus for the purpose of modifying the physical or chemical properties of the peptide for, e.g., ease of linking or coupling, and the like. Having identified different peptides of the invention, in some instances it may be desirable to join two or more peptides in a composition or admixture. The peptides in the composition can be identical or different, and together they should provide equivalent or greater reactivity than the parent peptide(s). The subject peptides find a variety of uses including preparation of specific antibodies. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984); Tam et al., J. Am. Chem. Soc. 105:6442 (1983); Merrifield, Science 232:341-347 (1986); and Barany and Merrifield, The Peptides, Gross and Meienhofer, eds., Academic Press, New York, pp. 1-284 (1979), each of which is incorporated herein by reference. Short peptide sequences, or libraries of overlapping peptides, usually from

about 6 up to about 35 to 50 amino acids, which correspond to the selected regions described herein, can be readily synthesized and then screened in screening assays designed to identify reactive peptides. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989); Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York (1987), and U.S. Pat. Nos. 4,237,224, 4,273,875, 4,431,739, 4,363,877 and 4,428,941, for example, whose disclosures are incorporated herein by reference.

As noted above, the invention provides antibodies which bind to and are specific for SHIP. The production of non-human antisera or monoclonal antibodies (e.g., murine, lagomorpha, porcine, equine) is well known and may be accomplished by, for example, immunizing an animal with SHIP protein or peptides. For the production of monoclonal antibodies, antibody producing cells are obtained from immunized animals, immortalized and screened, or screened first for the production of the antibody that binds to the SHIP protein or peptides and then immortalized. It may be desirable to transfer the antigen binding regions (i.e., F(ab')2 or hypervariable regions) of non-human antibodies into the framework of a human antibody by recombinant DNA techniques to produce a substantially human molecule. Methods for producing such chimeric and "humanized" molecules are generally well known and described in, for example, U.S. Patent No. 4,816,397; which is incorporated by reference herein in its entirety. Alternatively, a human monoclonal antibody or portions thereof may be identified by first screening a human B-cell cDNA library for DNA molecules that encode antibodies that specifically bind to SHIP according to

the method generally set forth by Huse et al. (Science 246: 1275-1281, 1989, which is incorporated by reference herein in its entirety). The DNA molecule may then be cloned and amplified to obtain sequences that encode the antibody (or binding domain) of the desired specificity.

The polynucleotide molecules, proteins and antibodies of the present invention provide methods for detecting genetic abnormalities at the SHIP locus. Such methods may be useful for genetic counseling, prognosis and staging of disease.

In another aspect of the invention, diagnostic methods and compositions are disclosed. Given the disclosed isolated polynucleotide molecules of the present invention and antibodies to SHIP, a variety of diagnostic assays are provided. The present invention provides a number of reagents that find use in assays to detect and/or quantitate levels of SHIP. Such reagents may be labeled with compounds that provide a detectable signal using conventional methods. Such labels which include, for example, chemiluminescers, paramagnetic particles, fluorophores, radionuclides, enzymes, enzyme substrates, and the like may be used in such assay methods to facilitate detection and/or quantitation of SHIP.

Antibodies against SHIP proteins may be used as reagents to detect wild-type and/or mutant SHIP in biological samples such as tumor biopsy samples, tissue and organ sections, peripheral blood cells and the like. Within other methods, antibodies of the present invention may be used in immunoassays to detect and/or quantitate SHIP. Immunoassays suitable for use in the present invention include, but are not limited to, enzyme-linked immunosorbant assays, immunoblots, inhibition or competition reactions, sandwich assays, radioimmunoprecipitation, and the like, as generally described in, e.g., U.S. Pat. Nos. 4,642,285; 4,376,110; 4,016,043; 3,879,262; 3,852,157; 3,850,752; 3,839,153; 3,791,932; and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, N.Y. (1988), each incorporated by reference herein.

Isolated DNA probes and PCR primers are designed as reagents for diagnostic assays for detecting the presence of SHIP or SHIP mutant sequences. The nature of the specific assay may depend on the type of mutational analysis to be carried out and the type of biological sample to be assayed. High molecular weight DNA may be obtained from suitable sources using commercially available kits. Commercially available kits include, the Genomic Isolation Kit A.S.A.P. (Boehringer Mannheim, Indianapolis, IN), Genomic DNA Isolation System (GIBCO BRL, Gaithersburg, MD), Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, NH), DNA Extraction Kit (Stratagene, La Jolla, CA), TurboGen Isolation Kit (Invitrogen, San Diego, CA), and the like. Use of these kits according to the manufacturer's instructions is generally acceptable for purification of DNA prior to practicing the methods of the present invention. PCR primers find used in the amplification of SHIP sequences from normal and diseased tissue. Such sequences may be analyzed by direct sequence analysis or by a variety of means to distinguish normal from mutant sequence. A comparison of fragment size and or comparison of sequences may be used to diagnose a number of diseases such as cancer. Within one example, SHIP-specific DNA probes are used in restriction fragment length polymorphism (RFLP) assays on DNA samples isolated from normal and diseased tissues to detect rearrangements and/or deletions of the SHIP gene. More subtle mutations may be detected by a variety of methods which include but are not restricted to single strand conformation polymorphism (SSCP) (Orita et al., Proc. Natl. Acad. Sci. USA 86: 2766-2770, 1989; which is incorporated by reference herein); dideoxy fingerprinting (ddf) (Orita et al., Genomics 5: 874-879, 1991 and Sarkar et al., Genomics 13: 441-443, 1992; which are incorporated by reference herein); restriction endonuclease fingerprinting (REF) (Liu and Sommer, BioTechniques 18: 470-477, 1995; which is incorporated by reference herein); PCR-based RNase protection assay (Murthy et al, DNA & Cell Biol. 14: 87-94, 1994; which is incorporated by reference herein) and

denaturing gradient gel electrophoresis (Fodde and Losekoot, Hum. Mutat. 3: 83-94, 1994). These methods rely on PCR amplification of coding regions within the genes of interest and use a variety of methods to distinguish between wild-type and mutant SHIP sequences. Within other methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon et al, Hum. Mutat. 3: 126-132, 1994). The present invention provides methods by which any or all of these types of analyses may be used. As disclosed herein, murine and human SHIP genes and cDNA have been cloned. Using these reagents, oligonucleotide primers may be designed to permit the amplification of sequences in the SHIP gene that may then be analyzed by either direct sequencing or other indirect methods such as SSCP to identify mutations within the SHIP gene.

The diagnostic and screening methods of the invention find use for individuals suspected of being at risk for developing a SHIP-associated or related disease such as cancer (e.g., family history of disease) or for patients in which such a screening is used to diagnose or eliminate SHIP-associated disease as a causative agent behind a patient's symptoms. In certain embodiments, methods for screening involve biological samples from the patient is provided (e.g., tissue biopsy and amniotic fluid samples) and the sample is screened for the presence of mutations in SHIP. Within these methods, a patient's SHIP gene is compared to normal SHIP DNA (i.e., wild-type SHIP) using a variety of methods, including RFLP analyses, SSCP, and the like, and mutations in SHIP are detected. An aberrant SHIP DNA size pattern, such as for RFLP analysis or SSCP analysis, aberrant SHIP protein and/or aberrant SHIP protein levels as determined by antibody assays would indicate that the patient has developed or is at risk to develop a SHIP-associated disease.

Prenatal diagnosis can be performed when desired, using a variety of methods to obtain fetal cells. These methods include, but are not limited to amniocentesis, chorionic villous sampling and fetoscopy. Prenatal analysis

of the SHIP gene is carried out using SSCP, RFLP, DDF and the like.

Also provided are kits and multicontainer units comprising reagents and components for practicing the assay methods of the present invention. Kits of the present invention may, in addition to reagents for detecting SHIP, contain enzymatic reagents such as reverse transcriptase or polymerase; suitable buffers; nucleoside triphosphates; suitable labels for labeling the reagents for detecting SHIP and developing reagents for detecting the signal from the label. In one aspect, kits of the present invention contain sequence-specific oligonucleotide primers for detecting polynucleotide molecules encoding SHIP. Such primers may be provided in separate containers or may be provided in combinations of one or more primer pairs in a series of containers. One aspect of the invention provides kits containing SHIP sequence-specific probes. Within yet another aspect, kits contain antibodies useful for detecting SHIP (or mutants thereof) in a sample. In addition to these components, the kits may also contain instructions for carrying out the assay and/or additional containers suitable for carrying out the reactions of the assay.

Within another embodiment of the invention, assays are designed for the screening of therapeutic compounds capable of replacing SHIP function or suppressing mutant SHIP function in cells containing SHIP mutants that result in abnormal cell proliferation. Within such assays, cells demonstrating abnormal cell proliferation due to a mutation in SHIP are exposed to test compounds. The cell proliferation of the treated cells are compared with the cell proliferation of untreated cells. Test compounds capable of replacing SHIP function or suppressing mutant SHIP function are those compounds that cause a reduction in the abnormal cell proliferation of the mutant cell.

Within another embodiment of the invention, the polynucleotide molecules encoding SHIP are used in screen for compounds that enhance or inhibit the interaction between SHIP

and its binding partners. In one embodiment, a yeast two hybrid screen, essentially as described in detail herein, is established in which the bait portion of the *Lex*A-bait hybrid protein is a SHIP-binding domain such as the PTB domain of Shc or the SH3 domain of Grb2 and the bait-binding portion of the bait-binding-VP16 hybrid protein contains at least the complementary binding domain of SHIP. Alternatively, the bait portion of the *Lex*A-bait hybrid protein is at least one binding domain of SHIP and the bait-binding portion of the bait-binding-VP16 hybrid protein is a SHIP-binding domain such as the SHIP-binding domains present in the Shc or Grb2. Binding domains of SHIP include the SH2 domain, the PTB domain and the SH3 domain. Test compounds that inhibit the interaction of the bait and bait-binding domains result in a decrease in reporter gene expression relative to the expression level in the absence of the test compound. Test compounds that enhance the interaction between the bait and bait-binding domains may be assessed by detecting or measuring an increase in the expression of the reporter gene. Such compounds may find use, for example, in re-establishing checkpoint control in cancer and other diseases associated with the loss of checkpoint control.

The polynucleotide molecules of the present invention may be used within the yeast two hybrid system described herein to screen for polynucleotide molecules capable of binding to other domains within SHIP. Within these embodiments, discrete binding domains of SHIP are used as bait in the *Lex*A-bait hybrid and a VP16 fusion library is screened for clones capable of binding to the SHIP binding domain in the bait hybrid by screening for clones capable of expressing the reporter gene(s).

In another aspect of the invention, animals, such as mice, and cell lines may be constructed that are heterozygous or homozygous for deletions of the SHIP genes. Such "knock-out" animals and cell lines may be useful as disease models and as test systems for therapeutics capable of overcoming SHIP deletion or mutations. The SHIP gene may be

deleted by homologous recombination using the method essentially set forth by Buerstedde and Takeda (Cell 67: 179-188, 1991; which is incorporated by reference herein). In a similar manner, homologous recombination may be used to delete the SHIP gene in pluripotent mouse embryonic-derived stem (ES) cells (Thomas and Capecchi, Cell 51: 503-512, 1987 and Doetschman et al., Nature 330: 576-578, 1987; which are incorporated by reference herein).

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1

Peptide Sequence Analysis

In initial but unsuccessful attempts to isolate polynucleotide molecules encoding SHIP, endogenous SHIP was isolated from mouse cells and subjected to sequence analysis. The peptide sequences were then used to screen libraries for cDNAs encoding SHIP.

To purify SHIP, murine 32D WT cells (Greenberger et al., Proc. Natl. Acad. Sci. USA 80: 2931-2935 (1983)) that had been infected with a retroviral vector encoding wild-type fms were grown at 37°C, 5% CO_2 and 95% humidity in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and WEHI conditioned medium which provided the IL-3 (see Lioubin et al., Mol. Cell. Biol. 14: 5682-5691 (1994); which is incorporated by reference herein in its entirety). Approximately 4-6 x 10^{11} cells were stimulated with a total of 8 x 10^6 Units of M-CSF for one minute at 37°C to stimulate tyrosine phosphorylation. The cells were lysed by incubation in lysis buffer (20 mM Tris (pH 7.5), 10 mM EDTA, 100 mM NaCl, 1 mM $ZnCl_2$, 0.05% NaN_3, 2% polyoxylethylene 9 lauryl ether (referred to hereinafter as $C_{12}E_9$), 2 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 mM iodoacetamide and 5 μg/ml leupeptin) for 15 minutes on ice. After lysis, the lysates were centrifuged at 25,000 x g for thirty minutes. The pellet

was discarded, and the lysate was loaded onto an anti-phosphotyrosine column prepared essentially as described by Schneider et al., J. Biol. Chem. 257: 10766-10769 (1982); which is incorporated by reference herein in its entirety) in load buffer (50 mM Tris (pH 7.5), 0.05% NaN_3, 150 mM NaCl, 2% $C_{12}E_9$). The column was washed with wash buffer (50 mM Tris (pH 7.5), 0.05% NaN_3, 300 mM NaCl, 0.1% SDS, 0.1% $C_{12}E_9$). The tyrosine phosphorylated proteins were eluted from the column by the addition of 100 mM phenyl phosphate in a buffer containing 50 mM Tris (pH 7.5), 0.05% NaN_3, 150 mM NaCl, and fractions were collected.

To cleave the protein for sequencing while reducing protein loss during processing, the 150 kD tyrosine phosphorylated protein was cleaved using the method essentially described by Patterson et al. (Anal. Biochem. 202: 193-203 (1992); which is incorporated by reference herein in its entirety). The column fractions were concentrated by 10% trifluoroacetic acid (TFA) precipitation followed by washes with 90% ethanol. The samples were then subjected to SDS-polyacrylamide gel electrophoresis on a 5% gel. The proteins were transferred to an IMMOBILON-CD transfer membrane (a cationic, PVDF membrane; Millipore Corporation, Bedford, MA) according to the manufacturer's instructions, and the membrane was stained using an IMMOBILON CD staining kit (Millipore) according the manufacturer's instructions. The protein band at 150 kD was excised.

The excised membrane was washed five times with MILLI-Q H_2O (Millipore) before the membrane was immersed in Digestion buffer (50 mM Tris-HCl (pH 8.5), 2 mM DTT). Endopeptidase Lys-C digestion was initiated by the addition of 1 μg of sequencing grade Endopeptidase Lys-C (Promega). The reaction mixture was sonicated for 5-10 seconds followed by an overnight incubation at 37°C. After overnight incubation, an additional 0.5 μg of Lys-C was added to the reaction mixture, and the mixture was incubated at 37°C for an additional eight hours. After the second incubation, 20 μl of 10% TFA in H_2O and 20 μl of CH_3CN, and the mixture was sonicated for 1-2

minutes. After sonication, the mixture was centrifuged at approximately 12,000 rpm in a tabletop microfuge. The supernatant was removed to a fresh tube, and the membrane was washed with 50 μl of 0.1% TFA, 10% CH_3CN. The fluid was removed from the membrane and combined with the first supernatant.

The combined supernatant was diluted with 0.1% TFA in H_2O to achieve a final CH_3CN concentration of no more than 3.5%. The sample was applied by multiple injections on an Ultrafast Microprotein Analyzer (Michrom BioResources, Inc.) fitted with a 1 x 150 mm Reliasil C18 column. The peptides were eluted over a one hour linear gradient from 5% B to 65% B (B = 70% C_H3CN, 30% 0.1% TFA H_2O) at a flow rate of 50 μl/min. Fractions were collected manually according to the absorbance at 214 nm. Selected fractions were subjected to Edman degradation on an ABI automated microsequencer model 477A (Applied Biosystems, Foster City, CA). The sequences of 14 peptides were determined. Eleven of the peptides, SEQ ID Nos:1-11, were found to be unique.

Degenerate primers corresponding to the peptides were prepared and used to screen for SHIP encoding cDNAs using numerous human and murine libraries, cDNAs and mRNAs with no success.

Example 2

Construction of an EML-C1 cDNA Library

A stem cell factor-dependent lympho-hematopoietic progenitor cell line (EML-C1; deposited under accession number CRL 11691 on July 25, 1994, at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852) was used as a cell source for the preparation of an EML-C1 cDNA library. The EML-C1 cell line was derived from bone marrow cells of male BDF_1 mice that had been infected with the retroviral vector containing the truncated retinoic acid receptor-alpha (RARα) cDNA and has been described in PCT

application WO 95/04142 (which is incorporated by reference herein). PolyA enriched RNA from EML-C1 cells was prepared. Following second-strand synthesis the ds-cDNAs were size-selected for cDNAs greater than 350 bp. The cDNAs were ligated to Not I linkers, and the linkered cDNAs were ligated into the Not I cloning site in the 2μ yeast expression vector pVP16 (Vojtek et al., Cell 74: 205-214 (1993) and Hollenberg et al., Mol. Cell. Biol. 15: 3812-3822 (1995); which are incorporated by reference herein in their entirety). The pVP16 vector contains the VP16 activation domain of Herpes simplex virus (HSV) under the control of the Saccharomyces cerevisiae alcohol dehydrogenase promoter; and both the LEU2 and Ampicillin-resistance selectable markers. Insertion of a DNA molecule of interest into the Not I site of the pVP16 vector resulted in expression of a VP16 hybrid protein having the protein of interest joined in-frame with VP16. The resultant cDNA library was termed the "VP16-EML library".

Example 3

Isolation of SHIP cDNAs

A. Isolation of a Partial Murine cDNA encoding SHIP

To overcome the inability to identify SHIP clones using traditional PCR amplification, a yeast two-hybrid screen described by Fields and Song (Nature 340:245 (1989) and U.S. Patent No. 5,283,173; which are incorporated by reference herein in their entirety), Vojtek et al. (ibid.), Hollenberg et al., (ibid.) and reviewed by Fields and Sternglanz (Trends Genet. 10: 286-292 (1994), was modified as described herein and used to screen the VP16-EML library described in Example 2. While the two-hybrid screen relies on the interaction between two hybrid proteins (the *Lex*A-bait protein and bait-binding protein-VP16 hybrids) to activate the expression of a reporter gene it is of limited use for proteins that require post-translational processing before binding can occur. The interaction between SHIP and Shc requires the phosphorylation

of SHIP. Thus, to use Shc as the bait portion of the *Lex*A-bait hybrid in a screen for binding with a SHIP-VP16 hybrid (the bait-binding-VP16 hybrid), the host cells must be capable of phosphorylating SHIP in the yeast host cell. To accomplish this, the yeast two hybrid screen was modified to include an additional expression unit that directed the expression of a tyrosine kinase. Three tyrosine kinases were tested for the ability to promote phosphorylation of a hybrid protein containing the full-length Shc joined to VP16 in a two hybrid system containing a *Lex*A-bait hybrid containing the Grb2 SH2 domain as the bait. The β-PDGF receptor cytoplasmic domain (amino acids 570-1106) was shown to provide the most effective tyrosine phosphorylation of target proteins fused to VP16. The *Lex*A-bait containing the phosphotyrosine-binding domain of Shc (Shc-PTB) was also tested for its ability to bind the EGFR tail with PDGFR as the tyrosine kinase.

The Shc-PTB domain was used as the bait portion of the *Lex*A-bait hybrid. The *Lex*A-Shc-PTB hybrid was capable of binding DNA but not activating transcription of a downstream reporter gene. The VP16-EML library (Example 2) provided a library of VP16 hybrid proteins containing candidate PTB-binding domains. The yeast two hybrid screen was used to identify members of the VP16-EML library capable of binding to the *Lex*A-Shc-PTB hybrid and therefore activating transcription of reporter genes. The S. cerevisiae host strain L40 contained multimerized *Lex*A binding sites inserted upstream of two reporter genes, the *HIS3* gene and the β-galactosidase gene. An expression unit comprising the ADH promoter joined to the PDGF receptor cytoplasmic domain (the tyrosine kinase) was inserted into the vector pBTM116 at the Pvu II site. The Shc-PTB domain (from amino acid 48 to 209 of Shc) was subcloned into the Sal I cloning site of the same vector such that the Shc-PTB and *Lex*A coding sequences were in-frame. The resulting plasmid pBTM116/PDGFR/Shc-PTB was co-transformed with Quiagen-purified (Quiagen, Chatsworth, CA) VP16-EML-cDNA library. Transformants were grown on medium lacking histidine, leucine, tryptophan, uracil, lysine and containing

50 mM 3-amino triazol to select for the presence of both plasmids (the *Lex*A-Shc-PTB plasmid and the VP16-EML-cDNA library plasmid). High levels of 3-amino triazol were essential to reduce false positives by reducing non-specific interactions between the *Lex*A and VP16 domains. Colonies capable of growing on the selective medium contained VP16-EML library clones that were capable of interacting with the *Lex*A-ShcPTB hybrid after tyrosine phosphorylation. Positive colonies from the transformation plates were grown in medium lacking uracil and leucine to cure the yeast cells of the pBTM116/PDGFR plasmid (containing the *Lex*A-Shc-PTB fusion and the tyrosine kinase). The cured yeast clones were then each mated to different AMR70 (Hollenberg et al., ibid.) yeast transformants containing pBTM116-based plasmids containing either *Lex*A alone, *Lex*A-Shc-PTB (no kinase), *Lex*A and the PDGFR expression unit (kinase), or pBTM116/PDGFR/Shc-PTB (*Lex*A-Shc-PTB and the PDGFR expression unit). As a control for specificity, a yeast strain transformed with an pVP16-based plasmid containing an EGF receptor tail-VP16 fusion was also mated with each of the AMR70 transformants. The diploids were then selected on medium lacking histidine, leucine, tryptophan, uracil, lysine and containing 50 mM 3-amino triazol. In addition, the colonies were scored for β-galactosidase activity. Only those clones that required both the presence of the tyrosine kinase and the Shc-PTB domain for growth under selection and that demonstrated β-galactosidase activity were selected for further analysis.

Plasmid DNA prepared from thirty-six positive transformants were subjected to Hae III restriction enzyme analysis. The plasmid DNA was also subjected to PCR amplification (35 cycles of (94°C for 15 seconds, 55°C for 15 seconds, 72°C for 2 minutes)) using primers derived from flanking sequences in the pVP16 vector. The PCR products from fourteen of the clones were subjected to DNA sequence analysis. All fourteen clones contained an NPXY motif [SEQ ID NO:14] known to bind the Shc-PTB domain. Ten of the fourteen clones appeared to be derived from the same gene, which

appeared to be unique. The deduced amino acid sequence of the overlapping cDNAs was shown to contain two of the peptides [SEQ ID Nos:7 and 11] determined by Lys-C digestion. This confirmed that the ten clones contained sequences encoding SHIP. The 1.1 kb cDNA insert from one clone, designated EML-11, was used as a probe of a northern blot of polyA-selected RNA from mouse FDC-P1 cells. The probe indicated a single mRNA species of approximately 5.0 kb.

B. Isolation of a Full-Length Murine cDNA encoding SHIP

The cDNA insert from clone EML-11 was random-primed and used as a probe to screen a combined polyA cDNA library constructed from murine FDC-P1 clone 19 (FDC-P1 cells that were infected with the Fms provirus produced by ψ2 cells; Lioubin et al., Mol. Cell. Biol. 14: 5682-5691 (1994)) and Mac11 cells (Gliniak and Rohrschneider; Cell 63: 1073-1083 (1990)) using the Stratagene UNIZAP EXPRESS kit (Stratagene Cloning Systems; La Jolla, CA) according to the manufacturer's instructions. Positive clones were subjected to secondary screens, tertiary screens and PCR amplification using primers designed from internal SHIP sequences. Ten positive clones were subjected to further analysis. Of the ten clones, two contained inserts of approximately 5.0 kb. One of the two clones, designated pBK-CMV-150.8, was sequenced in both orientations. Plasmid pBK-CMV-150.8 was deposited as an E. coli transformant at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, on September 12, 1995, under accession number ATCC 69898.

The insert present in pBK-CMV-150.8 contained a 4863 nucleotide insert with an open reading frame translating for a 1190 amino acid protein with a calculated molecular weight of 133,438 [SEQ ID Nos:12 and 13]. Of the two methionines present (positions 1 and 4) in the deduced amino acid sequence, the methionine at position 1 is believed to be the translational start given the presence of a SHIP peptide fragment [SEQ ID NO:6] corresponding to amino acids 2 through 9 that contained the methionine at position 4. In addition,

all eleven Lys-C peptides [SEQ ID NOs:1-11] derived from the native SHIP were distributed throughout the open reading frame (amino acids 2-9; amino acids 18-27; amino acids 66-73; amino acids of 307-322; amino acids 346-351; amino acids 355-358; amino acids 359-364; amino acids 514-523; amino acids 789-799; amino acids 866-871; and amino acids 1162-1171 of SEQ ID NO:13).

The amino-terminus of SHIP contains an SH2 domain (residues 8 to 105 of SEQ ID NO:13), and the carboxyl-terminus region contains two NPXY [SEQ ID NO:14] motifs (at amino acids 914-917 and 1017-1020 of SEQ ID NO:13) that are known to bind PTB domains. From the 10 clones identified using the two hybrid screen, three contained only the NPNY (amino acids 914-917, of SEQ ID NO:13) motif and seven clones contained both the NPXY motifs. The carboxy-terminal one-fourth of the molecule contains numerous proline rich clusters, of which at least three show homology to motifs known to bind SH3 domains. The deduced amino acid sequence also contained a GQPLHGKS [SEQ ID NO:15] motif seven amino acids C-terminal to the NPNY (amino acids 914-917, of SEQ ID NO:13) that shows homology with the conserved motif shown for ATP or GTP binding proteins.

Clone pBK-CMV-150.8 showed 55% identity from amino acids 188 to 856 of SEQ ID NO:12 with the human 51C gene product (Genbank Accession No. L36818) at both the amino acid and nucleotide level. Protein sequence comparisons with other proteins showed SHIP to have an IP-5P domain (amino acids 430-805 of SEQ ID NO:13) that shared homology with both type I and type II inositol phosphate 5-phosphatases. An alignment of the conserved amino acid sequences among inositol phosphate 5-phosphatases and SHIP (subdomains of the IP-5P domain) showed that the SHIP IP-5P domain contained signature sequences for inositol phosphate 5-phosphatases.

TABLE I. Alignment of conserved amino acid sequences among inositol phosphate 5-phosphatases and SHIP.

SHIP	535[a]	TSLGFVNSHL	553	RRNQNYMNI	584	LFWLGDLNY
51C[b]	491	TSFGFVNCHL	509	RRNQNYLDI	540	LFWFGDLNY
OCRL[c]	342	TTFCIVNSHL	360	RRNQDYKDI	392	VIWLGDLNY
IT5P2[d]	189	TSICVVNSHL	207	RRNQDYKDI	239	ILWLGDLNY

SHIP	636	EEEITFAPTYRF	671	PSWCDRVLWK	700	TSDHSPV
51C	590	EEEISFPPTYRY	624	PSWCDRILWK	653	TSDHSPV
OCRL	443	EGEIKFIPTYKY	470	PAWCDRILWR	496	TSDHKPV
IT5P2	270	EGELTFQPTYKY	301	PGVIG.FLWK	326	TSDHKPV

[a] The numbers at the left of each column denote amino acid position within each molecule [SEQ ID NO:13].

[b] 51C = human 51C gene product [SEQ ID NO:18].

[c] OCRL = human oculocerebrorenal gene product [SEQ ID NO:19].

[d] IT5P = human inositol triphosphate 5-phosphatase gene product [SEQ ID NO:20].

C. Isolation of a Human cDNA encoding SHIP

The cDNA insert from clone EML-11 was random-primed and used as a probe to screen a polyA cDNA library constructed from human HL-60 cells (Nature 270: 347-349 (1977)); available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 under accession number CCL-240) using the Stratagene UNIZAP kit (Stratagene Cloning Systems; La Jolla, CA) according to the manufacturer's instructions. The library was plated, and plaques were lifted onto maximum strength NYTRAN PLUS (Schleicher & Schuell, Keene, NH) membranes. The plaque lifts were then autoclaved for one minute and UV crosslinked with a Stratalinker (Stratagene Cloning Systems) UV Irradiator. The lifts were prehybridized for a minimum of three hours in prehybridization solution (50% deionized formamide, 4X SSC, 5X Denhardt's, 50 mM NaPi (pH 7.0), 0.5 mg/ml NaPPi, 0.1 mg/ml sheared salmon DNA, 1% SDS). The murine cDNA insert from clone EML-11 was random-primed using the READY-TO-GO (Pharmacia Biotech, Piscataway, NJ) kit according to the manufacturer's instructions. The lifts were removed from the prehybridization solution and hybridized for

a minimum of 18 hours with the ^{32}P-labeled SHIP cDNA in hybridization solution (50% deionized formamide, 4X SSC, 1X Denhardt's, 50 mM NaPi (pH 7.0), 0.5 mg/ml NaPPi, 0.1 mg/ml sheared salmon DNA, 1%SDS). The blots were washed at 55°C with 1X SSC, 0.1% SDS, then exposed to film at -70°C with intensifying screens. Positive clones were subjected to secondary screens and PCR amplification using primers designed from internal murine SHIP sequences. Among the clones identified, clone h9.1 contained an insert of approximately 4.2 kb. The insert was sequenced using DYEDEOXY TERMINATORS (Applied Biosystems, Foster City, CA). Sequence analysis confirmed that the clone contained sequences with high homology to the EML-11 clone, and comparison of the 5' end of this clone revealed that it did not contain the full-length sequence.

The HL-60 library was rescreened for a full-length cDNA using a PCR-amplified probe. The HL-60 library was subjected to PCR amplification (one cycle of 95°C for 1 minute, 35 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds), cooled to 4°C) using a sense primer designed to correspond to nucleotides 181 to 198 of SEQ ID NO:12 (from the murine SHIP), and an antisense primer (CGTTACCCATGTTCCAGG; SEQ ID NO:21) designed to complement to nucleotides 1320 to 1337 of the human SHIP nucleotide sequence [SEQ ID NO:26] to generate a radiolabeled probe. The library was probed essentially as described above with the exception that the hybridization was carried out at room temperature. Positive clones were subjected to secondary screens, and tertiary screens. The longest clone, designated h4.2-A2, contained an approximately 4.7 kb insert. Sequence analysis indicated that this clone contained more 5' sequence than the initial clone, but did not contain a full-length open reading frame.

To obtain the five-prime most end of the human SHIP DNA, the HL-60 library was amplified (one cycle of 94°C for 1 minute, 35 cycles of (94°C for 30 seconds, 57°C for 30 seconds, 72°C for 60 seconds), cooled to 4°C) using a sense T3

primer (CGAAATTAACCCTCACTAAAGGG; SEQ ID NO:22) and an antisense primer (GATGAGCTGGTCCAGCTTG; SEQ ID NO:23) complimentary to nucleotides 322 to 340 of the human SHIP sequence [SEQ ID NO:26]. Amplification products greater than 400 bp in length were pooled and ligated into pT7BLUE(R) vector (Novagen, Madison, WI). The ligation products were transformed into E. coli strain XL1-BLUE (Stratagene Cloning Systems). The transformants were screened using a blue/white beta-galactosidase screen. White colonies, indicating the presence of insert, were subjected to PCR amplification (one cycle of 96°C for 1 minute, 35 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes), one cycle of 72°C for 10 minutes followed by cooling to 4°C) using oligonucleotides (GGAAACAGCTATGACCATG; SEQ ID NO:24) and GGGTTTTCCCAGTCACGACGTTG; SEQ ID NO:25). The PCR products were electrophoresed in an agarose gel and products of greater than the expected 650 bp fragment size were eluted and subjected to sequence analysis. Sequence analysis indicated that the inserts contained a stop codon 5' to the initiation methionine of SHIP. Sequence analysis of the inserts and the clone h4.2-A2 gave an open reading frame of 3566 nucleotides with a deduced amino acid sequence of 1189 amino acids [SEQ ID NOS:26 and 27]. The 4.7 kb insert from clone h4.2-A2 was used as a digoxigenin-labeled probe of fixed metaphase spreads by FISH karyotyping essentially as described by Trask et al. (Am. J. Hum. Genet. 48: 1-15 (1991); which is incorporated herein by reference in its entirety). FISH analysis indicated that human SHIP maps to human chromosome 2q37.

Example 4

Expression of Recombinant Ship

The entire insert of the pBK-CMV-150.8 clone, which includes both the 5' and 3' untranslated region including the polyA tail, was subcloned into the retroviral vector LXSN (Miller and Rosman, Biotechniques 7: 980-990, (1989); which is

incorporated herein by reference in its entirety). The SHIP vector and an empty LXSN vector were each transfected into Psi2 packaging cells (Mann et al., ibid.) and selected in Dulbecco's modified Eagles medium containing 10% calf serum and 0.5 mg/ml G418 to permit packaging of the vector. The resultant retrovirus was used to infect Rat2 cells (ATCC CRL 1764). The infected Rat2 cells were grown in Dulbecco's modified Eagles medium containing 10% fetal bovine serum. Western analysis of lysates of cells containing the SHIP vector demonstrated a 150 kD protein in contrast to cells containing an empty vector.

The role of SHIP in Fms signaling was examined by overexpressing the SHIP protein in FDC-P1 cells (FD) and in FDC-P1 cells transformed with Fms (FD-Fms). FDC-P1 cells were originally described by Duhrsen and Metcalf (Leukemia 2: 329-333, (1989); which is incorporated herein by reference in its entirety) and a clone (clone 19) was selected for its ability to differentiate along the macrophage lineage after retroviral expression of Fms and growth in M-CSF (Bourette et al., Growth Diff. 6:631-645, (1995); which is incorporated herein by reference in its entirety). FDC-P1 and FD-Fms were each infected by co-culture over Psi2 cells infected with either empty vector (LXSN) or the SHIP vector. The infected cells were selected for approximately 2 weeks in the presence of G418. After selection SHIP expression and growth in soft agar were examined.

Factor-dependent growth was measured in 1 milliliter agar cultures with modification of the methods described by Rohrschneider and Metcalf (Mol. Cell. Biol. 9: 5081-5092, (1989); which is incorporated herein by reference in its entirety). Briefly, one volume of 2x Iscove's medium (3.54 g Iscove's powder (Sigma, St. Louis, MO), 0.605 g sodium bicarbonate, 20 ml fetal bovine serum and 200 U/ml penicillin + 200 micrograms/milliliter streptomycin in 100 ml of water) was mixed with one volume of 0.62% agar at 42°C. The cells were added to the agar mixture in a small volume of Dulbecco's Modified Eagles (DME) containing 10% fetal bovine serum, and

one milliliter of each mixture was plated onto a non-treated 33 mm plastic culture dish. Each assay was performed in quadruplicate. Appropriate growth factors were added to the bottom of the dishes prior to the addition of cells. After 7-10 days, the colonies were counted and cultures were photographed. The average number of colonies for each culture condition (four plates) was calculated with the standard error. No differences in SHIP expression or cellular behavior were detected between cells infected with the empty vector and cell infected with the SHIP vector.

Under the assumption that negative selection for Ship-expressing cells occurred during the first two weeks in G418 selection, the soft agar assays were repeated with the exception that the cells were analyzed either immediately after co-culture over the Psi2 cells by placing them in agar assay with G418 or by examining growth of the infected cells after a brief three-day selection in a high concentration of G418. Both methods gave similar results. FDC-P1 cells infected with both empty virus (FD-Lx) and FDC-P1 cells infected with Ship vector (FD-Ship) did not grow without added growth factor or with the addition of M-CSF because the parent cell line lacked the M-CSF receptor. In the presence of IL-3, the FD-Lx cells formed large colonies and FD-Ship cells formed fewer, and in general, smaller colonies. FD-Fms cells infected with empty vector (FD-Fms/Lx) produced colonies of the size and number seen previously for FD-Fms cells (Rohrschneider and Metcalf, ibid.). In contrast, the FD-Fms/Ship cells (FD-Fms cells infected with Ship vector) formed fewer and significantly smaller colonies than the control FD-Fms/Lx cells grown in the presence of M-CSF. When the FD-Fms/Ship cells were grown in IL-3 the colony size was similar to the control FD-Fms/Lx cells but a reduction in colony number was observed. Although FD-Fms/Ship cells grown in the presence of M-CSF formed mainly small colonies in soft agar, a few were larger and compact like those formed in the presence of IL-3. These larger colonies presumably lack the retrovirally expressed SHIP yet were G418 resistant and

accounted for the outgrowth of "normal" cells and lack of Ship overexpression after longer term culture. These results indicated that overexpression of Ship in FDC-P1 cells results in a suppression of both M-CSF, and to a lesser degree, IL-3 dependent growth.

The expression level of Ship was examined in both FD-Fms/Lx and FD-Fms/Ship cells after infection by 2-day growth over Psi2 cells followed by a three day selection in 0.75 milligram/milliliter G418. Immunofluorescence detection of the SHIP protein with affinity purified antibodies demonstrated that the FD-Fms/Ship cells expressed slightly higher levels of the protein than the endogenous Ship detected in the FD-Fms/Lx cells. These results contrasted sharply with the relative ease of Ship overexpression in Rat2 and Psi2 cells and indicated that expression levels are very tightly regulated in the FDC-P1 cells and that overexpression may have deleterious effects. The growth inhibitory role of Ship and its inositol 5 phosphatase activity in Fms signaling is opposite that reported for the positive mitogenic effects of PI-3 Kinase activity.

Example 5

Purification of Ship

Polyclonal rabbit antibodies to murine SHIP domains were raised to GST fusion proteins containing either amino acids 670-688 of SEQ ID NO:12 or amino acids 889-1046 of SEQ ID NO:12. Antisera to the C-terminal GST fusion were affinity purified by antigen affinity chromatography.

The affinity purified antisera against the unique C-terminal region of SHIP (amino acids 889-1046 of SEQ ID NO:12) recognized a major 150 kD protein in hematopoietic (32D-Fms) cells that co-migrated with the tyrosine phosphorylated protein observed after M-CSF stimulation (Lioubin et al., Mol. Cell. Biol. 14: 5682-5691, (1994); which is incorporated herein by reference in its entirety). The 150kD SHIP band was

the most prominant but often appeared as a doublet, and multiple minor bands (up to five) below the main protein were always detectable. All of the SHIP proteins contained phosphotyrosine. In addition, immunoprecipitation with anti-SHIP antibodies and blotting with anti-Shc antibodies demonstrated that the tyrosine phosphorylated $p52^{Shc}$ (and a lesser amount of $p45^{Shc}$) was associated with SHIP after M-CSF stimulation. The amount of tyrosine phosphorylated SHIP was elevated after treatment with M-CSF and correlated with increased amounts of associated Shc. Moreover, treatment of SHIP immunoprecipitates with 100 mM phenylphosphate resulted in the elution of Shc, indicating that such associations are dependent on SHIP phosphorylation. These results indicated that antibodies to the SHIP protein sequence (SEQ ID NO. 13) recognized the same 150kD protein previously described (Lioubin et al., ibid.) and that the protein becomes tyrosine phosphorylated and associated with Shc after M-CSF stimulation of 32D-Fms or FD-Fms cells.

Normal rabbit serum, anti-Ship antiserum and anti-Shc antiserum were each used to immunoprecipitate quiescent or M-CSF-stimulated FDC-P1/FMS cells. Briefly, cell lysates were prepared and incubated with the primary antisera and 10 microliters of protein G-agarose bead matrix (SEPHAROSE; Pharmacia Biotech, Inc., Piscataway, NJ) for one hour at 4°C. The beads were washed five times with wash buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% polyoxyethylene 9 lauryl ether ($C_{12}E_9$), 200 micromolar orthovanadate). Proteins were eluted by the addition of 50 microliters of 2X SDS-polyacrylamide gel electrophoresis sample buffer at 95°C for five minutes, and the beads were removed by centrifugation.

Example 6

5-Phosphatase Assays

To assess whether Ship has 5-phosphatase activity, 5-phosphatase assays were carried out on murine Ship immunoprecipitates. Briefly, radiolabeled phospholipids were

first prepared in a kination reaction with phosphotidyl inositol 3-kinase (PI3 Kinase). PI3 Kinase was prepared by immunoprecipitation from Psi2 cells (Mann et al., Cell 33: 153-159, (1983); which is incorporated herein by reference in its entirety) using a rabbit polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY). The PI3 Kinase-containing immune pellets were washed six times in kinase buffer (50 mM Hepes (pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$), and 25 microliter portions were aliquoted into microfuge tubes. Radiolabeled PtdIns(3*,4)P_2 and PtdIns(3*,4,5)P_3 were prepared by first drying 100 micrograms each of PtdIns(4)P and PtdIns(4,5)P_2 (Sigma, St. Louis, MO) under argon. The dried material was resuspended in 100 microliters of kinase buffer by sonication for five minutes on ice in a batch sonicator. Each lipid suspension was added to an aliquot of the PI3 Kinase immune pellet. The reaction was initiated by the addition of ^{32}P-γ-ATP (Dupont/NEN) (at 3,000 Ci/mmol) to a final concentration of 150 nM. After an incubation for one hour at room temperature, 100 microliters of chloroform/methanol)(1:1 v:v) was added to each reaction, and the organic phase of each reaction containing the radiolabeled phospholipids was washed twice with 200 microliters of 2M KCl. The radiolabeled phospholipids were used immediately or were stored under argon at -70°C.

Approximately 5 x 10^5 cpm each of radiolabeled phosphatidyl inositol 3,4 phosphate (PtdIns(3*,4)P_2) and radiolabeled phosphatidyl inositol 3,4,5 phosphate (PtdIns(3*,4,5)P_3) in chloroform/methanol were individually evaporated under argon and resuspended in 100 microliters of 5-Pase buffer (50 mM Hepes (pH 7.5), 10 mM $MgCl_2$). The resuspended radiolabeled lipids were added to aliquots of normal rabbit serum, anti-SHIP or anti-Shc immunoprecipitates prepared from quiescent or M-CSF stimulated FDC-P1/Fms cells. The reactions were stopped after an incubation of 20 minutes at room temperature by extracting the phospholipids with an equal volume of chloroform/methanol and 100 microliters of 2M KCl.

After extraction, the reaction products were separated on Silica Gel 60 TLC plates that had been treated with 1% potassium oxalate in 50% ethanol and allowed to dry at least 24 hours at room temperature before use. The TLC plates were developed as described by Traynor-Kaplan et al. (Nature 334: 353-356, (1988); which is incorporated by reference herein in its entirety) using a solvent mixture of chloroform/acetone/methanol/glacial acetic acid/water (80:30:26:24:14 - v:v:v:v). Radiolabeled phospholipids were detected by radiography. The identity of the phospholipids was obtained by comparison of chromatographic mobility on the same TLC plate. The assays demonstrated that SHIP immunoprecipitated from quiescent or M-CSF stimulated FDC-P1 cells hydrolyzed PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 with equal efficiency. An increase in the 5-phosphatase activity was observed in Shc immunoprecipitates only after M-CSF stimulation. This is in agreement with the observation that SHIP association with Shc occurs only after M-CSF stimulation. In contrast, PtdIns(3,4)P_2 was not a substrate for SHIP.

In addition, endogenous murine SHIP was shown to release phosphate from inositol (1,4,5) phosphate as detected in a Malachite Green assay (Harder et al., Biochem. J. 298: 395-401, 1994; which is incorporated by reference herein in its entirety)using inositol polyphosphates, Ins(1,4,5)P_3 and Ins(1,3.4,5)P_4 (Boehringer Mannheim, Indianapolis, IN), as substrates were performed in the same 5-Pase buffer.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the claims.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide encoding mammalian SHIP polypeptide or a sequence which is complementary thereto.

2. The isolated polynucleotide of claim 1, which is cDNA.

3. The isolated polynucleotide of claim 1, which encodes the polypeptide of SEQ ID NO:13 or SEQ ID NO:27.

4. The isolated polynucleotide of claim 3, which is substantially identical to the sequence of SEQ ID NO:12 or SEQ ID NO:26 or is complementary thereto.

5. An isolated DNA construct for expression of mammalian SHIP polypeptide, which comprises:
a) a transcriptional promoter,
b) an isolated DNA sequence which encodes the mammalian SHIP polypeptide or a biologically active fragment thereof, and
c) a transcriptional terminator, each operably linked for expression of the SHIP polypeptide.

6. The isolated DNA construction of claim 5, wherein the DNA sequence encodes the polypeptide of SEQ ID NO:13 or SEQ ID NO:27.

7. The isolated DNA construct of claim 6, wherein the DNA sequence is substantially identical to the polynucleotide sequence of SEQ ID NO:12 or SEQ ID NO:26.

8. The isolated DNA construct of claim 5, wherein the biologically active fragment binds Shc.

9. A method for producing isolated mammalian SHIP polypeptide, comprised:

cultivating a host cell into which has been introduced a DNA construct which comprises an operably linked transcriptional promoter, an isolated DNA segment encoding the mammalian SHIP polypeptide or a biologically active fragment thereof, and a transcriptional terminator, under suitable conditions to allow the expression of the mammalian SHIP polypeptide encoded by the DNA segment; and

isolating the mammalian SHIP polypeptide from the host cell.

10. The method according to claim 9, wherein the host cell is a mammalian or fungal cell.

11. A cultured eukaryotic cell transformed or transfected with a DNA construct which comprises the following operably linked elements:

a transcriptional promoter;

an isolated DNA sequence encoding a mammalian SHIP polypeptide or a biologically active fragment thereof; and

a transcriptional terminator.

12. The eukaryotic cell of claim 11, which is a mammalian cell.

13. The eukaryotic cell of claim 12, which does not express endogenous mammalian SHIP polypeptide.

14. The eukaryotic cell of claim 11, wherein the biologically active fragment binds Shc.

15. An isolated probe which comprises an oligonucleotide capable of specifically hybridizing with a gene which encodes mammalian SHIP or allelic and species variants thereof.

16. The isolated probe of claim 15 which comprises from about 40 to about 60 nucleotides in length.

17. The isolated probe of claim 15, which is labeled to provide a detectable signal.

18. The isolated probe of claim 15, which comprises an oligonucleotide of 15 or more contiguous nucleotides of SEQ ID NO:12 or its complement or SEQ ID NO:26 or its complement.

19. A purified antibody which binds specifically to purified mammalian SHIP polypeptide or a biologically active fragment thereof.

20. The purified antibody of claim 19, wherein the polypeptide has a sequence identical to SEQ ID NO:13 or SEQ ID NO:27.

21. The purified antibody of claim 19, which is a monoclonal antibody.

22. A method for determining the presence of SHIP polypeptide in a biological sample, which comprises incubating the sample with an antibody which specifically binds to SHIP under conditions sufficient for immune complex formation and determining the presence of immune complexes.

23. The method of claim 22, wherein the antibody is a monoclonal antibody or purified antiserum.

24. A method for detecting the presence of or predisposition to develop SHIP associated disease in a subject, the method comprising evaluating the characteristic SHIP nucleic acid sequence in a sample from the subject in relation to wild-type SHIP nucleic acid sequence, and thereby detecting the presence of or predisposition to develop SHIP associated disease in the subject.

INTERNATIONAL SEARCH REPORT

Inter onal application No.
PCT/US96/14754

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/23.1; 435/6, 69.1, 240.2; 530/350, 388.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/6, 69.1, 240.2; 530/350, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLU EMBASE MEDLINE BIOSIS INPADOC DISSABS SCISEARCH LIFESCI
search terms: inositol phosphatase, SHIP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,E	WARE et al. Cloning and Characterization of Human SHIP, the 145-kD Inositol 5-Phosphatase That Associates With SHC After Cytokine Stimulation. Blood. October 15, 1996. Vol. 88. No. 8 pages 2833-2840.	1, 2, 5, 8-17, 19, 22-24
X, P	DAMEN et al. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. Proc. Natl. Acad. Sci. USA. February 1996. Vol. 93 pages 1689-1693.	1, 2, 5, 8-17, 19, 22-24
A,E	ONO et al. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc RIIB. Nature. September 19, 1996. Vol. 383 pages 263-266.	1, 2, 5, 8-17, 19, 22-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 DECEMBER 1996	Date of mailing of the international search report 27 DEC 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer AMY ATZEL, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inter onal application No.
PCT/US96/14754

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MAJERUS, P. W. Inositols do it all. Genes and Dev.01 May 1996. Vol. 10 pages 1051-1053.	1, 2, 5, 8-17, 19, 22-24
X, P	LIOUBIN et al. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. Genes and Dev. May 1996. Vol. 10 pages 1084-1095.	1, 2, 5, 8-17, 19, 22-24

INTERNATIONAL SEARCH REPORT

Inter onal application No.
PCT/US96/14754

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3, 4, 6, 7, 18, 20 and 21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the nucleotide and peptide sequences recited in the claims as SEQ ID NOs 12, 13, 26, and 27 were not found anywhere in the specification.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US96/14754

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/02, 21/04;C12Q 1/68; C12P 21/06, 21/08; C12N 5/00; C07 K 1/00, 14/00, 16/00, 17/00